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13. ABSTRACT (Maximum 200 Words)

My research is focused on the BRCA2 protein, whose mutations have been implicated in the development of breast, ovarian, male breast, prostate, pancreatic cancers and Fanconi anemia. It is intended to elucidate some of the biological functions of BRCA2 and/or regulation of its in vivo function through generation/utilization of new reagents and identification of new BRCA2 interacting proteins. During this second year of grant support, I was able to identify a completely novel protein, named CLB2 in this study, as a major physiological partner of BRCA2. I discovered that CLB2 is a chromatin bound protein and is required for BRCA2's chromatin binding. In light of these findings, it is attempting to speculate that disruption of CLB2 function would lead to significant impairment of BRCA2's tumor suppressor function realized at least in a large part through its DNA recombination/repair activity which presumably requires its docking to the chromatin.

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INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among women and is the No. 1 health threat to women in the United States and other western countries. One eighth of western women develop breast cancer during their lifespan. A significant proportion of breast cancer cases, in particular those arising at a young age, are clustered in certain families that have germ line mutations in either of two breast cancer susceptibility genes, *brca1* and *brca2*. My research is focused on the BRCA2 protein, mutations of which have been implicated in the development of breast, ovarian, male breast, prostate, pancreatic cancers, and Fanconi anemia. Human BRCA2 is a very large protein consisting of 3,418 amino acids, approximately twice the size of BRCA1. It is essential for embryonic development, given that mice deficient of BRCA2 die before birth. It is also essential for homologous recombination and DNA repair, at least in part through controlling the function of the Rad51 recombinase (Chen et al. 1998 and Davies et al., 2001). BRCA2 mutant cells reveal gross genomic instability and sweeping chromosomal abnormalities, including broken chromosomes and various unnatural, illegitimate chromosomal structures. Normal cells slow their DNA synthesis after S phase DNA damage to allow time for DNA repair, while BRCA2 deficient cells show radio resistant DNA synthesis (RDS) without efficient repair, which likely facilitates the accumulation of replication errors and potentially accelerates cancer formation (see Venkitaraman 2002 for review). A small fraction of the pool of BRCA2 and BRCA1 molecules exists in a common biochemical protein complex and also colocalizes in small nuclear "foci" during S-phase and several hours after DNA damage, implying that the two breast/ovarian cancer tumor suppressors participate in a common DNA damage respose/repair pathway (Chen et al., 1998). This information notwithstanding, how BRCA2 operates as a tumor suppressor and, in particular, inhibits breast cancer development is largely unappreciated.

BODY (STRATEGIES AND PROGRESS)

The study of BRCA2 function has been hampered by a lack of high quality immune detection reagents and, in part, by its extraordinary size. The goal of my research is to elucidate key elements of the as yet unappreciated biochemical and biological function of BRCA2. In particular, I have focused on attempting to achieve this goal by generating a set of new, monospecific BRCA2 Abs and using them to search for novel, BRCA2 interacting proteins. This approach is beginning to bear fruit as will be described below..

During the first calendar year of my fellowship, I focused my efforts on generating a collection of new BRCA2 antibodies and searching with them for new BRCA2 interacting proteins. First, I generated a series of rabbit polyclonal antibodies against different segments of human BRCA2. Among them, one, anti-F8, has proven to decorate S-phase and DNA damage-induced BRCA2 nuclear foci. This Ab is highly BRCA2-specific, since its nuclear staining signal can be completely eliminated by pre- treatment of a given target cell line with BRCA2 specific siRNA. No such signal depletion was noted after exposure of the cells to a control RNAi reagent. The use of F8 has enabled me to track the nuclear localization of at least a subpopulation of BRCA2 protein under various conditions.

Second, using a FLAG-HA double tagging strategy, I was able to identify Tip48, a putative ATPase/DNA helicase, as a novel BRCA2- associated protein. Unfortunately, the results of subsequent experiments suggest that, despite the monospecificity of F8, the BRCA2-Tip48 interaction is too weak to study in detail.

In the past (second) calendar year, I have continued the hunt for new BRCA2-interacting proteins with additional BRCA2 Abs. After testing multiple methods and conditions, I have succeeded in identifying a novel protein as a major BRCA2 partner in the nucleus. The current report is focuses on this discovery.

1. Identification of CLB2 as a novel BRCA2-intercating protein. In an attempt to discover new components of endogenous BRCA2-containing protein complexes, whole Hela cell extract was immunoprecipitated using a new BRCA2 monoclonal antibody bound to protein A Sepharose beads. After IP, the beads were washed extensively and then analyzed by SDS-PAGE followed by silver staining. As shown in Figure 1A, a number of distinct protein bands were evident. Subsequent mass spectrometry analysis confirmed that the highest molecular weight protein was BRCA2 and the 40KD band was Rad51, an established and important BRCA2-binding protein. Interestingly, in addition to BRCA2 and Rad51, a relatively abundant 130KD protein was also present in the IP. The protein was identified by mass spectroscopy as a "hypothetical" protein-i.e. the product of an assumed, long ORF in the human genome that had, otherwise, not been studied by others. Bioinformatic analysis of this polypeptide revealed no significant sequence homology with any other known protein and no conserved domain structures. Based on the results of subsequent studies of the potential function of this protein, I have termed it "CLB2", which stands for Chromatin Loader of BRCA2.

2. Cloning the *clb2* gene. The putative CLB 2 mRNA sequence was obtained from the public database and used to design primers for an RT-PCR amplification of its cDNA. As expected, an RT-PCR reaction amplified a 3.6KB DNA fragment from the RNA of T98G glioblastoma cells, indicating that this mRNA is expressed. This DNA fragment was cloned into a TA cloning vector, sequenced, and found to correspond to the predicted cDNA sequence.

3. Generation of antibodies against CLB2. The full length cDNA was also cloned into the pET42 GST-fusion vector and its product synthesized in *E.coli*. Although the mRNA product was partially degraded in bacteria, a relatively abundant polypeptide product (fused to0 GST) was readily purified and then used to immunize rabbits. A polyclonal Ab developed and was shown to recognize a protein of the expected (130KD) size in lysates of multiples cell lines (not shown). Furthermore, this antibody efficiently coimmunoprecipitated endogenous BRCA2, while the preimmune serum from the same rabbit produced no BRCA2 signal under the same conditions. These data (see Fig. 1A) strongly suggest that that CLB2 is a normal binding partner of endogenous BRCA2.

4. Analysis of CLB2-BRCA2 interaction. Following the extraction of cells with a low salt + detergent-containing buffer (20mM Tris-Cl pH7.5, 100mM NaCl, 1mM EDTA, 0.5% NP40), BRCA2 can be separated into two distinct species that migrate slightly differently in SDS gels (unpublished). As shown in Figure 2A, the majority of BRCA2

protein was soluble in the low salt- extracted fraction [so-called S100 (compare lanes 1 and 2)]. The remaining, insoluble BRCA2 could be partially solubilized by nuclease treatment or nearly completely extracted with higher salt (420mM in this experiment). These two fractions were designated "SMN" (lane 4) and "S420" (lane 6), respectively. In contrast to BRCA2, only a small fraction of CLB2 was present in the S100 fraction. Like BRCA2, the low salt- insoluble form of CLB2 could also be partially solubilized by nuclease digestion (lane 4) and almost completely solubilized by high salt (i.e. 420 mM; lane 6).

Since both BRCA2 and CLB2 in the NETN-insoluble pellet could be partially solubilized by nuclease treatment, one can hypothesize that some CLB2 is chromatin bound and interacts with BRCA2 in that context. The data do not allow one to conclude that this is the case for all possible BRCA2/CLB2 complexes, however.

5. Effect of CLB2 loss of function on the chromatin association of BRCA2. Since it is possible that CLB2 interacts with BRCA2 in chromatin, we wondered whether its association with this fraction was BRCA2-dependent and/or vice versa. To answer this question, I first treated cells with CLB2 siRNA and examined the effect of CLB2 knockdown on the nuclear staining pattern of BRCA2. As shown in Figure 3A, in control siRNA treated cells, a significant amount of the BRCA2 in nuclei was concentrated in nuclear foci that also contained BRCA1. The specificity of the BRCA2 staining pattern was confirmed by the results of BRCA2 siRNA which practically eliminated all BRCA2 nuclear staining without affecting the BRCA1 staining of dot structures. Notably, CLB2 siRNA treatment also greatly reduced the BRCA2 staining signal without affecting BRCA1 staining. I am attempting to generate additional CLB2 antibodies, both polyclonal and monoclonal, with the goal of further testing the validity of this observation.

Furthermore, as shown in Figure 3B, two, different CLB2 siRNAs each significantly reduced the abundance of BRCA2 in the pellet fractions arising during 200 and 400 mM salt extraction of cells (compare lanes 2, 3, 4 and lanes 1, 5, 6). An analogous experiment aimed at learning whether CLB2 expression affects the abundance of BRCA2 in the 100 mM NaCl insoluble fraction is in progress.

As a key control here, it will be important to know whether the total abundance of BRCA2 in nuclei is affected by CLB2 knock down (as measured by standardized western blotting) or whether its concentration in dots (and in chromatin pellets) falls while its overall intracellular abundance does not. In the latter instance, one could argue that CLB2 function affects BRCA2/ nuclear dot association (and BRCA2/chromatin association) without affecting total BRCA2 expression. The preliminary data shown in Fig 3 lead one to the sense that CLB2 knockdown does not lead to a global reduction in whole cell BRCA2 abundance. However, this will need to be tested rigorously in my ongoing experiments. If CLB2 depletion does not \lead to a global reduction in BRCA2 abundance but still suppresses its association with nuclear dots and with chromatin, this would imply that CLB2 functions, in part, by promoting the association of BRCA2 with chromatin, a novel function.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified CLB2, a novel protein, as a significant BRCA2-interacting protein
- Cloned the gene encoding CLB2
- Generated a CLB2 polyclonal antibody
- Made the following observations
  1. CLB2 is a chromatin bound protein.
  2. CLB2 interacts with BRCA2, primarily in chromatin.
  3. CLB2 expression controls the association of BRCA2 with chromatin. Whether or not it affects total BRCA2 intracellular abundance remains to be determined.

REPORTABLE OUTCOMES: N/A

CONCLUSIONS

During this second year of grant support, I have again made significant progress. Remarkably, I was able to identify, clone, and initially characterize a novel protein, CLB2, as a significant intracellular partner of BRCA2. I have found that CLB2 is a chromatin- bound protein that appears to be required for BRCA2- chromatin association. Whether this is a specific CLB2 effect on chromatin- associated BRCA2 or whether CLB2 affects total BRCA2 abundance/expression with a secondary effect on BRCA2/chromatin binding remains to be determined.

Whatever the case, in light of these findings it is attempting to speculate that disruption of CLB2 function would lead to significant impairment of BRCA2 DNA recombination/repair function, a process that almost surely requires the docking of BRCA2 in chromatin.

In light of these observations, as a primary goal, ongoing experiments are aimed at learning whether the effect of CLB2 expression on BRCA2/ chromatin association is specific to chromatin-bound BRCA2 or is a secondary effect of controlling overall intracellular BRCA2 expression. Secondly, I wish to understand the mechanism by which CLB2 affects BRCA2 abundance and/or its selective association with chromatin. The third goal is to learn whether CLB2 controls BRCA2 DNA repair function. Finally, it will be important to know whether the CLB2/BRCA2 interaction is clinically relevant. In particular, is loss of CLB2function/expression a characteristic present in a significant subset of a sizeable collection of sporadic,. BRCA2 +/+ breast carcinomas?

A manuscript describing the CLB2/BRCA2 interaction and its functional significance is expected within the next calendar year.

REFERENCES

Chen J, Silver DP, Walpita D, Cantor SB, Gazdar AF, Tomlinson G, Couch FJ, Weber BL, Ashley T, Livingston DM, Scully R. (1998) Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol Cell* 2: 317-28.

Chen PL, Chen CF, Chen Y, Xiao J, Sharp ZD, Lee WH (1998) The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. *Proc Natl Acad Sci USA* 95:5287-92.

Davies AA, Masson JY, McIlwraith MJ, Stasiak AZ, Stasiak A, Venkitaraman AR, West SC (2001) Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol Cell* 7:273-82.

Venkitaraman AR (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 108: 171-82.

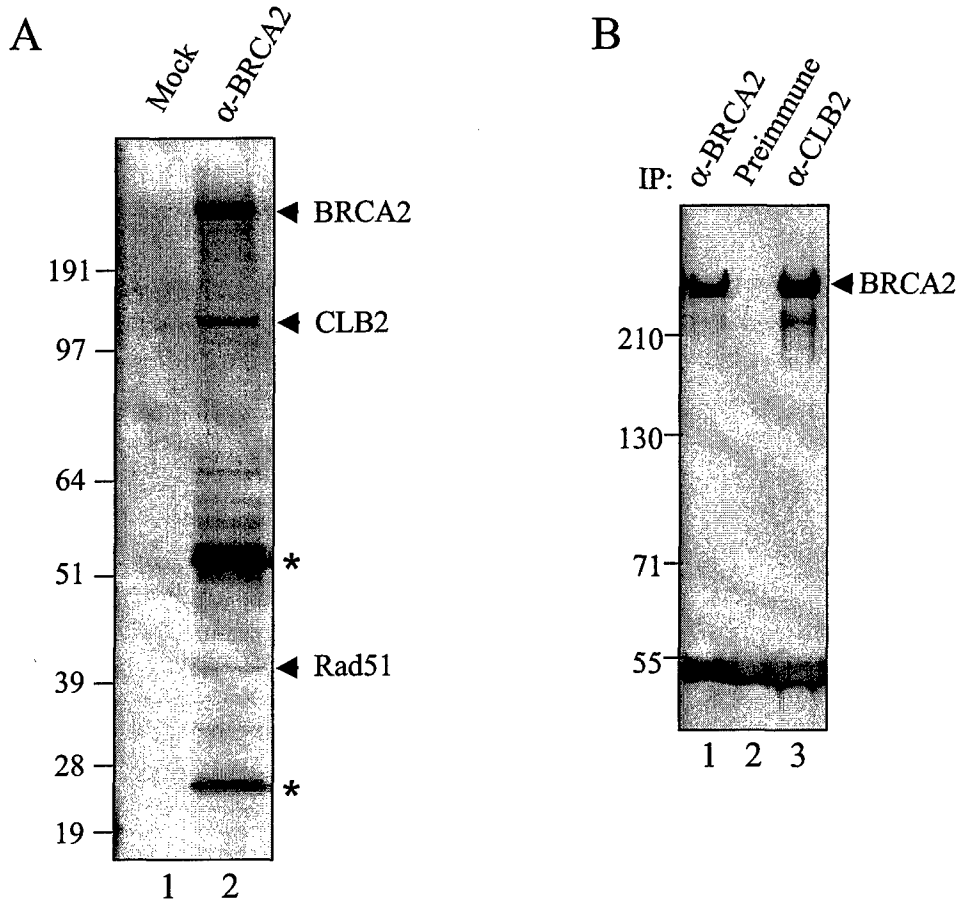

Figure 1.CLB2 is a major physiological partner of BRCA2. (A) Identification of CLB2. Hela whole cell extract (WCE) was immunoprecipitated using a mouse monoclonal BRCA2 antibody and stained with silver. In mock preparation, only protein A sepharose beads but no antibody was added. A novel 130KD protein, named CLB2, was detected in addition to Rad51, a known BRCA2 interacting protein. The heavy and light chains of IgG are ndicated by asterisks. (B) Whole cell extract of 293 cells were immunoprecipitated with rabbit antisera against BRAC2 (lane1) and CLB2 (pane 3). The CLB2 preimmune serum was used as a negative control (lane 2). The immuno-precipitates were subsequently probed for BRCA2 using a monoclonal antibody.

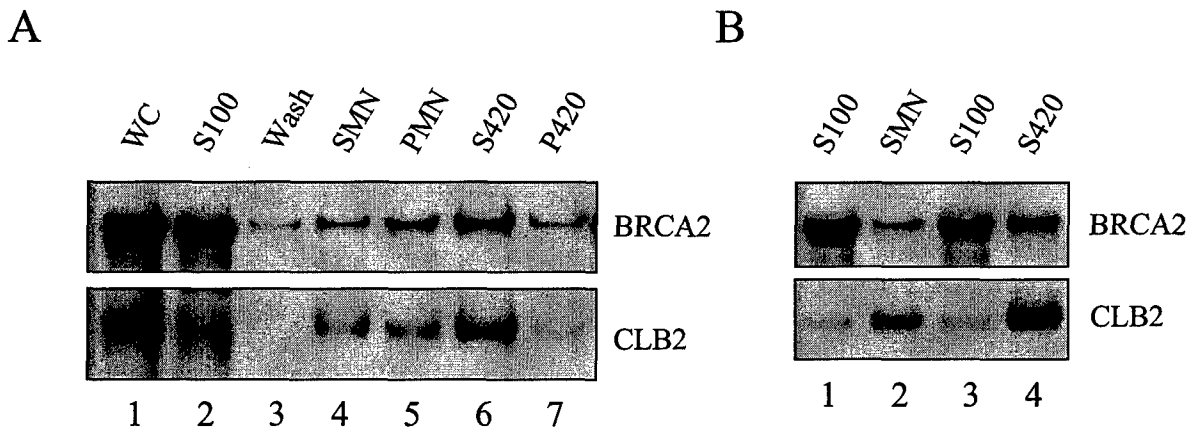

Figure 2. CLB2 interacts with BRCA2 in chromatin. (A) Localization of BRCA2 and CLB2 in different cellular fractions. U2OS cells were trypsinized, divided into three aliquots and pelleted. One cell pellet was immediately frozen and saved as a whole cell aliquot (WC, lane 1). The second cell pellet was extracted for 15 min with low salt (100mM NaCl) NETN buffer on a rocker in the cold room and the extracted material (supernatant) was taken as S100 (lane 2); then, the pellet was washed with micrococal nuclease buffer and spun down, the supernatant was saved as "Wash" (lane 3) and the remaining pellet was digested with micrococal nuclease for 15min at room temperature. The resulting solubilized chromatin was designated SMN (lane 4) and the remaining pellet was labelled PMN (lane 5). The third U2OS cell pellet was first extracted the with 100mM NETN. The resulting pellet was further extracted with high salt (420mM NaCl) NETN buffer and then centrifuged. The resulting supernatant and pellet were designated S420 (lane 6) and P420 (lane 7), respectively.
The volume of buffers used in all above steps was kept the same and all three pellets (WC, PMN, P420) were resuspended in the same volume of low salt NETN buffer and sonicated before SDS sample buffer was added. As a result, each lane contains protein derived from equivalent numbers of cells. (B) Association of BRCA2 with CLB2 in different protein fractions. S100 and SMN from the second cell pellet as well as S100 and S420 from the third cell pellet were immunoprecipitated with a monoclonal BRCA2 antibody .The presence of BRCA2 and of CLB2 in the relevant precipitates was determined by western blotting.

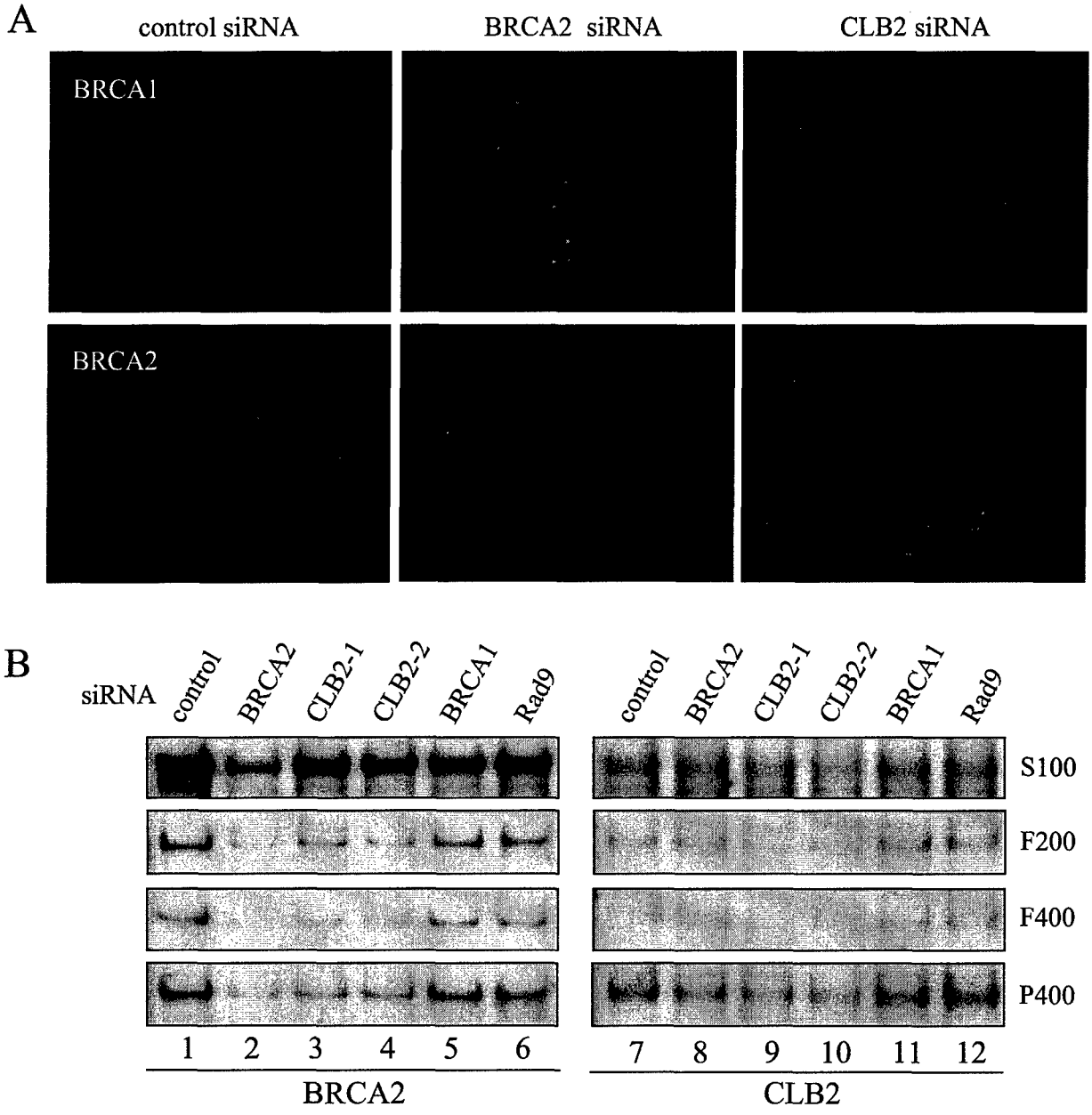

Figure 3. CLB2 contributes to the chromatin association of BRCA2. (A) U2OS cells were treated with control (luciferase) siRNA or siRNAs targeting BRCA2 and CLB2. 48 hr after transfection, cells were fixed and stained with a pair of antibodies against BRCA1 (SD118 mAb) and BRCA2 (anti-BRCA2F8 polyclonal antibody). (B) U2OS cells were treated with two different CLB2 siRNAs as well as other siRNAs as indicated and collected 48 hr after transfection. Cell pellets were first extracted with low salt NETN and S100 fractions were obtained. Then the remaining pellets were extracted with same volume of 200mM NETN. The extracted supernatants were designated F200 and the pellets were further extracted with same volume of 400mM NETN which resulted in F400 (supernatants) and P400 (pellets). The final pellets were resuspended in the same volume of 400mM NETN buffer and sonicated. An equal volume of each fraction was loaded for western blotting to detect BRCA2 and CLB2.